UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
NATIONAL HEALTH AND ENVIRONMENTAL EFFECTS RESEARCH LABORATORY
Research Triangle Park, NC 27711

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OFFICE OF
RESEARCH AND
DEVELOPMENT

MEMORANDUM:

SUBJECT: Internal Report Identifying Effects of Atrazine on Luteinizing Hormone

FROM: Ralph L. Cooper, Chief, MD-72
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THRU: Hal Zenick,
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Attached please find a report authored by Ralph Cooper et al., describing the effects of the chlorotriazines herbicide Atrazine on the pre-ovulatory surge of luteinizing hormone in the rat, an endocrine event necessary for normal ovulation. As you are aware, several studies have shown that atrazine and atrazine metabolites will inhibit the estrogen-, or estrogen plus progesterone-induced surge of luteinizing hormone in the ovariectomized female rat. Furthermore, atrazine has been shown to alter the spontaneous surge of LH released during the afternoon of vaginal proestrus in the intact female rat. These studies have typically employed a 3- or 4-day oral dosing period with the hormone measured on the last day of dosing. To date, the LOEL or NOEL for the atrazine-induced suppression of the LH surge has not been identified. To our knowledge, the lowest published oral dose of atrazine found to suppress LH secretion is 6.25 mg/kg (Cooper *et al.*, 2007), administered to the intact, Long-Evans female once a day for four consecutive days. Again, in that study a NOEL was not identified. This report summarizes studies conducted to confirm the Cooper *et al.*, 2007 study and identify a NOEL. In addition, this report provides a more detailed description of the experimental procedures employed in this work.

Enc: Internal Report

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Summary Report:

Evaluating the Effect of the Chlorotriazine Herbicide Atrazine on the Amplitude of the Pre-ovulatory LH surge in the Long-Evans Rat.

By
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This Internal Report was prepared for the use by the Office of Pesticide Programs, Office of Chemical Safety and Pollution Prevention. July 1, 2010

Abstract:

Several studies have shown that atrazine and atrazine metabolites will inhibit the estrogen-, or estrogen plus progesterone-induced surge of luteinizing hormone in the ovariectomized female rat. Furthermore, atrazine has been shown to alter the spontaneous surge of LH released during the afternoon of vaginal proestrus in the intact female rat. These studies have typically employed a 3- or 4-day oral dosing period with the hormone measured on the last day of dosing. To date, the LOEL or NOEL for the atrazine-induced suppression of the LH surge has not been identified. To our knowledge, the lowest published oral dose of atrazine found to suppress LH secretion is 6.25 mg/kg (Cooper *et al.*, 2007), administered to the intact, Long-Evans female once a day for four consecutive days. Again, in that study a NOEL was not identified. This report summarizes studies conducted to confirm the Cooper *et al.*, 2007 study and identify a NOEL. In addition, this report provides a more detailed description of the experimental procedures employed and identifies a NOEL of 1.56 mg/kg when the LH surge was analyzed at several different time points on afternoon of vaginal proestrus. In addition, when the 1800 h peak values were analyzed, a NOEL of 3.12 mg/kg was identified.

Background:

The effects of atrazine (ATR) and its metabolites on mammalian health have been studied extensively, with the most consistent effects being changes in the hormonal control of the female reproductive axis (Cooper *et al.* 2000; Eldridge *et al.* 1999). Oral gavage exposure to ATR disrupted estrous cyclicity (Cooper *et al.* 1996) and diminished the surge of luteinizing hormone (LH). For example, in ovariectomized rats, atrazine and its metabolites have been shown to decrease the estrogen- or estrogen- plus progesterone- induced LH surge (Cooper *et al.*, 2000, McMullin *et al.* 2004, Foradori *et al.*, 2009). In general, these studies have employed relatively high doses of atrazine (i.e., 50–300 mg/kg/day administered for a period of 3–4 days). Using a similar ovariectomized, estrogen-primed model, Morseth *et al.*, (1996) reported significantly lower doses of atrazine were required to decrease the LH surge in females treated for a period of 6 months prior to measuring the hormone. This led to the belief that if the animal was exposed to atrazine for a longer duration, the lowest observed effect level (LOEL) or no observed effect level (NOEL) would be lower than that observed in an acute or short-term exposure study. This was supported in part by the observation that a single treatment with 300 mg/kg atrazine was required to lower the LH surge in ovariectomized, estrogen-primed Long-Evans (LE) rats (Cooper *et al.* 2000). We also found that this dose was without effect on similarly treated Sprague-Dawley (SD) rats. In contrast, three daily doses of atrazine (50-300 mg/kg) did reduce the estrogen-induced LH surge in LE but not SD females, indicating that multiple days of exposure appeared to be necessary to affect LH secretion and that the SD strain was less sensitive to the effects of atrazine on LH. Atrazine was found to suppress the estrogen-induced LH surge in ovariectomized SD females following a 21-day exposure, but with a NOEL and LOEL of 75 mg/kg and 150 mg/kg, respectively. Again, a LOEL was not identified in the LE females after 21 days, further indicating a strain difference in sensitivity to this compound.

In a study of the intact, regularly cycling female, Cooper et al (2007), reported that oral atrazine exposure for four consecutive days (one estrous cycle) reduced the spontaneous, pre-ovulatory surge of LH with a LOEL of 6.25 mg/kg. In that study, we tested atrazine doses of 6.25, 12.5, 25 and 75 mg/kg. The 75 mg/kg dose was selected because it was identified as the dose that would marginally interfere with regular ovarian cycles in the four-day cyclic female treated for two weeks. We hypothesized that a dose below 75mg/kg would alter the LH surge, but it was somewhat surprising that a NOEL was not identified at the doses tested. Subsequent to the publication of that manuscript, we repeated the evaluation of atrazine on the LH surge in regularly cycling females and compared vehicle-treated animals with those exposed to 6.25, 3.12 and 1.56 mg/kg. The results of this work are reported below.

Methods

All experiments were approved by the U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory's (NHEERL) Institutional Animal Care and Use Committee and were conducted in accordance with National Institute of Health standards for laboratory animal research. The research was conducted within the IRP entitled: "Effects of Selected Pesticides and their Metabolites on Female Reproduction and the Neuroendocrine System in Rats". NHEERL-H-RTP/RTD-EB-RLC-2007-01-r0. This research was also reviewed and included as a part of the Office of Research and Development, EPA Multi-Year Plan for Human Health, Long Term Goal 1 (mode and mechanism of action), Annual Performance Goal (APG 2012-01) and Annual Performance Measure APM 148.

Animals

Female Long-Evans Hooded rats (60 days old) were purchased from Charles River Laboratories (Raleigh, NC) and housed singly in a room maintained at 22 ± 2°C with a 14:10h light:dark schedule (lights on at 0500 hours: lights out at 1900 hours). Purina Laboratory Rat Chow 5001 and water were provided *ad libitum*. Beginning at 80 days-of-age, the ovarian cycle of each female was monitored for a period of 2 weeks by evaluating daily vaginal lavages as described in detail by Cooper *et al.* (1993). Only females displaying regular, 4-day estrous cycles during the two-week pretreatment period were included in the experiments.

Dosing solutions and procedures

Atrazine (purity 97.1%) was a gift from Syngenta Crop Protection, Inc. (Greensboro, NC). All dosing was administered by oral gavage in a suspension of 1% methyl cellulose/distilled water (M-7140, Sigma Chemical Co., St. Louis, MO) in a volume of 5.0 ml dosing solution / kg body weight. Control animals received 1% methyl cellulose vehicle (5.0 ml / kg body weight) only.

Experimental Procedures

To evaluate the pituitary-ovarian hormone response to ATR exposure over the course of one estrous cycle (e.g., 4 consecutive days), females were gavaged once per day at 0900 hours beginning on the day of vaginal estrus and ending on the following day of proestrus.

Animals assigned to treatments were ranked by body weight and placed into control or atrazine groups such that the mean body weights for all groups were similar. The experiment was conducted in three blocks. Animals in the first block received either vehicle, 25 or 75 mg/kg. As a NOEL was not established, three additional groups of animals, receiving either 25, 12.5 or 6.25 mg/kg, were subsequently tested in a second block, along with a concurrent control group. These data were analyzed and published by Cooper *et al.* (2007). However, as the 6.25 mg/kg dose group was still not a NOEL, a subsequent experiment was conducted in which two additional dose groups were examined (3.12 mg/kg and 1.56 mg/kg) along with a concurrent control and another 6.25 mg/kg group. Thus, for each block, concurrent controls were included, along with the lowest dose from the previous block. The number of animal in each group is presented in the results, as each animal included in the data analysis had to meet three criteria at the time of kill: (1) a proestrous vaginal smear, (2) increased uterine weight and (3) elevated concentration of progesterone.

To fully evaluate the spontaneous ovulatory surge of LH, animals from each dose group were killed by decapitation at 1200, 1400, 1600, 1800 or 2000 hours (h). The animal was removed from its cage to an adjacent necropsy room, where it was decapitated. This process was routinely accomplished within 10-15 seconds from the time the home cage was opened. After decapitation, the trunk blood was collected on ice in 16-x 100-mm serum-separation tubes (Becton-Dickinson, Rutherford, NJ) and centrifuged at 3000 rpm for 30 min at 4°C. Serum was subsequently divided into two aliquots and stored at -80°C until assayed for hormone concentrations.

Radioimmunoassay

Radioimmunoassay for serum LH was performed using kit materials obtained from the National Hormone and Pituitary Program through Dr. A. Parlow (UCLA, Los Angeles, California). Tracer iodination with ^{125}I was performed in-house, and the assays conducted according to recommendations provided, with the sensitivity optimized by a 24 h co-incubation of sample and first antibody prior to the addition of ^{125}I-labeled tracer. The sensitivity of the LH assay is 10 pg/ml and the intra- and inter-assay coefficients of variation were 7.7% and 5.9% respectively. Serum progesterone for each animal was analyzed using a Coat-A-Count Progesterone Kit (Coat-A-Count, Siemens Healthcare Diagnostic, Deerfield, IL) that was performed according to the manufacturer's instructions with an assay sensitivity of 0.1 ng/ml and an intra- and inter- assay coefficient of variation of less than 10%.

Statistics

Comparisons of the number of animals achieving the criteria set for a proestrous female were done using a Chi square test. Comparisons of the hormone values in this experiment were conducted using a one-or two-way analysis of variance followed by the appropriate t-tests comparing individual groups (Graph Pad Prism 4, (La Jolla, CA). with the significance level set at a probability of less than 0.05.

Results

Identification of proestrus females: The original goal was to achieve a minimum sample size of 8 rats per dose/time treatment group, this aim was not achieved for all cells because the number of animals that actually met the criteria for achieving proestrus varied as the experiment progressed. In order for the female to be classified as proestrus the animal had to have a proestrous vaginal smear, have a uterine weight of greater than 500 mg, and for the later sample times (1600-2000 h), elevated serum progesterone. These criteria are based on our laboratory's extensive experience evaluating the female rat.

A total of 470 females displaying regular 4-day cycles were selected from a larger pool of animals. Of those selected, 351 animals (for all dose/time groups) actually met all three criteria defining proestrus. Figure 1 depicts the percentage of females in each dose group that achieved proestrus. For each dose group the number of females not meeting the criteria ranged between 10.4-38.6% with the greater percentage of excluded animals being in the two higher dose groups (figure 1). A chi square analysis revealed that a significantly greater proportion of females did not meet the outlined criteria in the 75 mg/kg dose. Although there was a loss of animals across all groups a sufficient number of animals within all but two groups (1.56 mg/kg at 1400 h and 1600 h) met these criteria.

Uterine weight: Of the animals that met the established criteria for being proestrus, there was no difference among the time by dose groups in uterine weight. This was true for both absolute uterine weights and for the weight of this tissue when corrected for body weight. Figure 2 depicts the absolute weight at 1800 h. A similar lack of difference was noted at all time points tested.

While Figure 2 demonstrates that the absolute uterine weight of the females was not different across the group. Table 1 depicts the relative uterine weight expressed as mg/100g body weight. Table 1 also shows the actual numbers of animals used in each of the dose x time groups for which the LH surge was analyzed. Note that an N=2 was present in the 1.56 mg/kg dose group at 14 and 1600 h. This low number was the result of the small number of animals meeting the criteria for proestrus. Because the LH values for these animals did not influence the outcome of the overall data analysis and the LH values of the animals receiving a greater dose of atrazine revealed no difference in LH at this time point, it was felt that increasing the N would not be a wise use of resources.

Serum Progesterone: In control animals, as expected serum progesterone revealed a dramatic increase over the course of the day of proestrus (Figure 3) with the highest concentration observed at 2000h.

The concentration of serum progesterone observed for each treatment group at each time point was not different than that observed in the control groups for any of the dose groups (Figure 4) when the concentration of this hormone for all the time points measured was analyzed. However, when the progesterone concentration for 1800 hour groups was analyzed separately, the 25 mg/kg/day group was statistically lower than the controls (Figure 5).

Serum LH: As described in the methods section, this experiment was conducted in three consecutive blocks. Therefore, the control groups from each of the three blocks were compared to identify any potential block effects. Table 2 depicts the number of observations, mean and variance (SEM & SD) of each of these groups and shows that all three groups of vehicle treated animals were not statistically different. Thus the control groups were combined for the subsequent statistical analysis. It should also be noted that a comparison of the treated groups with their concurrent control revealed the same statistical difference at each dose x time level.

Effect of atrazine on the LH surge: Figure 6 shows the results of treatment with atrazine for four consecutive days on the LH surge observed on the day of vaginal proestrus. The peak LH values in control and each of the atrazine-treated groups were observed at 1800 h, indicating that the onset of the surge was not modified. Analysis of LH secreted in the control vs. the treated females revealed a significant suppression of LH at 3.12 mg/kg/day and above. This analysis identified 1.56 mg/kg/day as the NOEL.

By way of comparison, these data extend and support the earlier published data comparing the effect of atrazine on the LH surge in intact females by Cooper *et al.*, (2007). However, as mentioned previously, the 2007 study did not identify a NOEL (Figure 7).

Comparison of the1800 h LH values only: Because the 1800 h time point represented the peak for all dose groups tested, this time point was analyzed separately by one-way ANOVA. This type of analysis indicates that the LOEL for 1800 h was 6.25 mg/kg/day and the NOEL was 3.12 mg/kg/day (Figure 8).

Discussion:

The results of this study confirm and extend our understanding of the dose required to alter the amplitude of the pre-ovulatory surge of LH in the rat. In this study, we selected a high dose of 75 mg/kg. This dose was based on the results of our previous observation (Cooper *et al.*, 1996) that it would disrupt, but not completely abolish ovarian cycles in the LE female. In the present study, the 75 mg/kg/day dose resulted in a significant decrease in the number of females that met the criteria for classification as proestrus on the day of kill. This observation indicates that four days of treatment at this dose was sufficient to modify the pituitary gonadal axis in the female controlling ovarian function. Importantly, doses of atrazine that were substantially lower than 75 mg/kg were also found to significantly reduce the peak amount of LH secreted during the pre-ovulatory surge. Thus, we found that the peak concentration of LH was statistically lower in females dosed at concentrations as low as 3.12 mg/kg or 6.25 mg/kg depending on the way the surge was analyzed (i.e., 2-way ANOVA for entire day vs. one-way ANOVA at 1800 h).

The observation that doses as low as 1.56 or 3.12 mg/kg/day for 4 days will lower peak LH concentrations demonstrates that the amount of atrazine required to modify the LH surge is lower that what had been previously believed. However, it should be noted that a NOEL/LOEL dose was generally not determined in the previous, short-term evaluations of the effect of atrazine on LH in ovariectomized females (i.e., Cooper *et al.*, 2000; McMullin *et al.* 2004 and Foradori *et al.*, 2009). Using this model, the only study in which a NOEL for a 3-day exposure was identified was that of Cooper et al (2000). In that study, a LOEL of 150 mg/kg and a NOEL and 75 mg/kg was identified in the Sprague-Dawley female. This was not the case for the identically treated LE female. In this strain, all doses tested suppressed the surge (lowest dose = 75 mg/kg) and thus a LE female NOEL was not identified. These results also indicate that the LE female is more sensitive than the SD female to the short-term effects of atrazine on LH in the ovariectomized model and perhaps in the intact female as well. In the intact, regularly cycling female, Cooper *et al.* (2007) reported that a dose of 6.25 mg/kg/day for 4-day (the same treatment period in the current study), significantly reduced LH at 1800 h on vaginal proestrus. The data in the present study confirm this finding and identify a NOEL.

The mechanisms underlying the change in LH observed in the present study are still not understood. It is interesting that doses less than 300 mg/kg administered on the day of an expected LH surge in the estrogen-primed, ovariectomized female was without effect on the amount of LH released. Similarly, when proestrous females were exposed to a single dose of atrazine on the morning of vaginal proestrus, it did not affect ovulation in the LE female. Why a single large dose is without effect while four-days of dosing in the same strain will decrease serum LH at doses as low as 3.12 mg/kg remains to be determined.

Literature cited:

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McMullin, T. S., Andersen, M. E., Nagahara, A., Lund, T. D., Pak, T., Handa, R. J., and Hanneman, W. H. Evidence that atrazine and diaminochlorotriazine inhibit the estrogen/progesterone induced surge of luteinizing hormone in female Sprague-Dawley rats without changing estrogen receptor action. Toxicol Sci 79, 278-86, 2004.

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Table 1: Relative Uterine Weight (mg/100 g bw) of Females Included in Proestrous LH Surge Experiment

	Control		1.5 mg/kg		3.12 mg/kg		6.25 mg/kg	
Time	Weight	N	Weight	N	Weight	N	Weight	N
1200	235.4 ± 11.4	18	211.4 ± 15.8	5	238.2 ± 16.8	4	243.4 ± 14.7	8
1400	278.3 ± 12.8	13	231.4 ± 10.3	2^	238.0 ± 16.9	5	278.4 ± 20.9	12
1600	257.3 ± 15.7	14	243.5 ± 43.3	2^	256.7 ± 8.7	4	276.0 ± 20.4	6
1800	297.2 ± 13.5	27	295.9 ± 20.2	11	313.3 ± 11.2	12	322.9 ± 17.7	17
2000	299.3 ± 14.6	18	313.2 ± 42.0	5	275.0 ± 31.0	5	318.4 ± 15.4	8
			12.5 mg/kg		25 mg/kg		75 mg/kg	
			N	Weight	N	Weight	N	
1200			227.9 ± 12.0	11	233.8 ± 17.1	8	261.6 ± 21.3	8
1400			274.1 ± 15.7	6	260.6 ± 14.5	14	245.7 ± 13.6	6
1600			258.5 ± 11.7	6	298.1 ± 12.4	11	269.2 ± 14.6	6
1800			297.3 ± 19.5	7	289.5 ± 11.4	14	296.9 ± 20.2	8
2000			332.9 ± 17.7	9	316.5 ± 15.9	13	292.5 ± 13.3	7

* N= the number of animals included in the LH analysis at each dose and time point.

^ Groups for which a sufficient number of animals were not observed.

TABLE 2: Serum LH means (ng/ml) in Control Animals from Three Different Blocks			
Observation	**Block 1**	**Block 2**	**Block 3**
1	33.36	14.29	20.29
2	8.40	20.25	17.91
3	23.85	26.21	11.93
4	28.41	13.03	15.52
5	19.63	17.75	14.80
6	28.13	24.03	28.52
7	18.96	28.44	18.85
8	29.54	15.30	20.66
9			20.39
10			24.6
11			24.54
Mean	23.785	19.913	19.82
Standard Deviation	7.949	5.786	4.823
Number	8	8	11
SEM	3	2.187	1.454

Figure Legends

Figure 1. Percentage of Females Not Meeting Criteria for Proestrus. This is the percentage of rats for each dose examined collapsed across different time points.

Figure 2. Absolute uterine weight for females killed at 1800 h.

Figure 3 Serum Progesterone in Control females.

Figure 4. Serum progesterone in females dosed with atrazine (mg/kg) and killed at time points noted. There were no statistical differences between control and treated animals at any of the time points. Note that the 1.5 mg/kg group killed at 1400 h and 1600h contained only two animals each.

Figure 5. Serum progesterone in all females killed at 1800 h. Asterisk indicates that the concentration of this hormone in the 25 mg/kg dose group was significantly below that of the controls. However, the progesterone for the 75 mg/kg was not statistically different.

Figure 6. Serum LH in atrazine-treated proestrous females. Asterisk depict groups that are statistically different from control at the same time point using a 2-way ANOVA and Bonferroni posttests. Note that the 1.56 dosed animals only had an N=2 at the 1400 and 1600 h time points and thus these data were not included in the analysis.

Figure 7. Effects of atrazine on serum LH published by Cooper *et al.*, 2007.

Figure 8. Serum LH levels at 1800 h.

Figure 1:

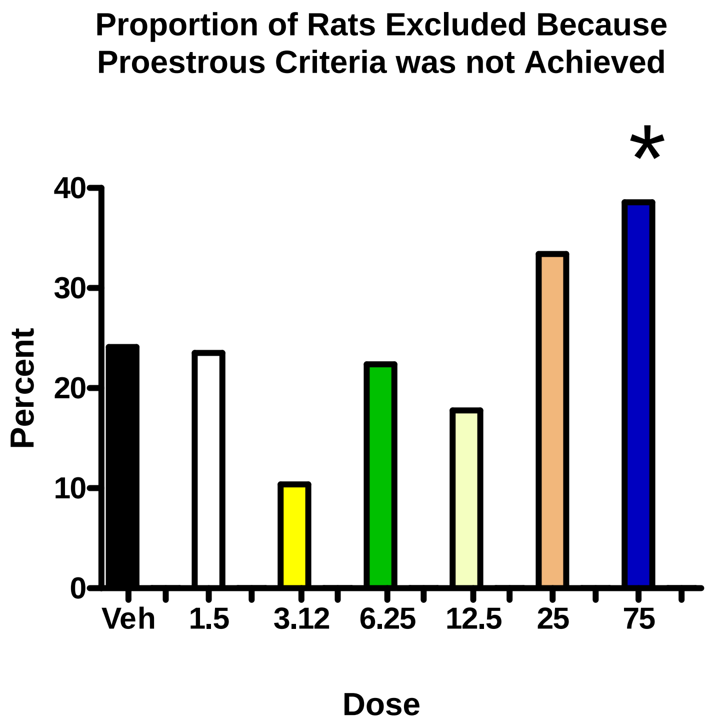

Figure 2

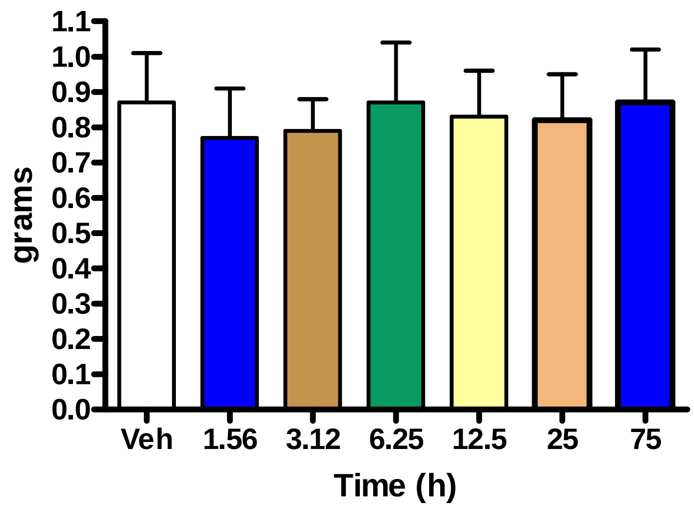

Figure 3.

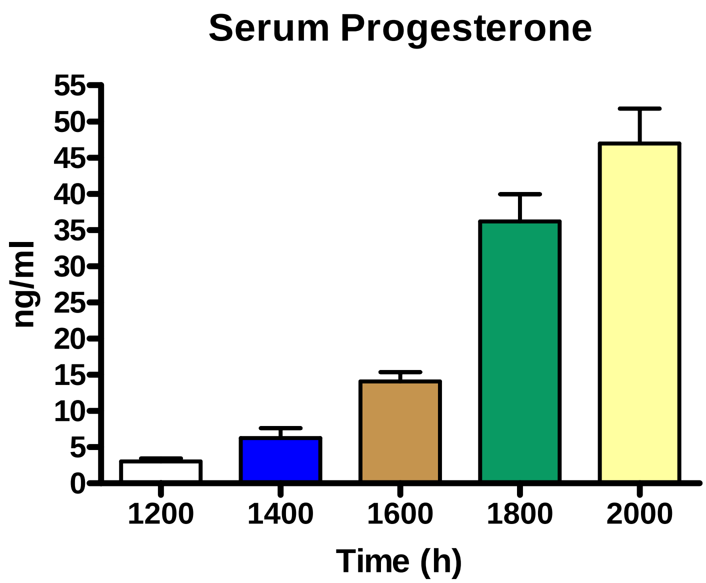

Figure 4

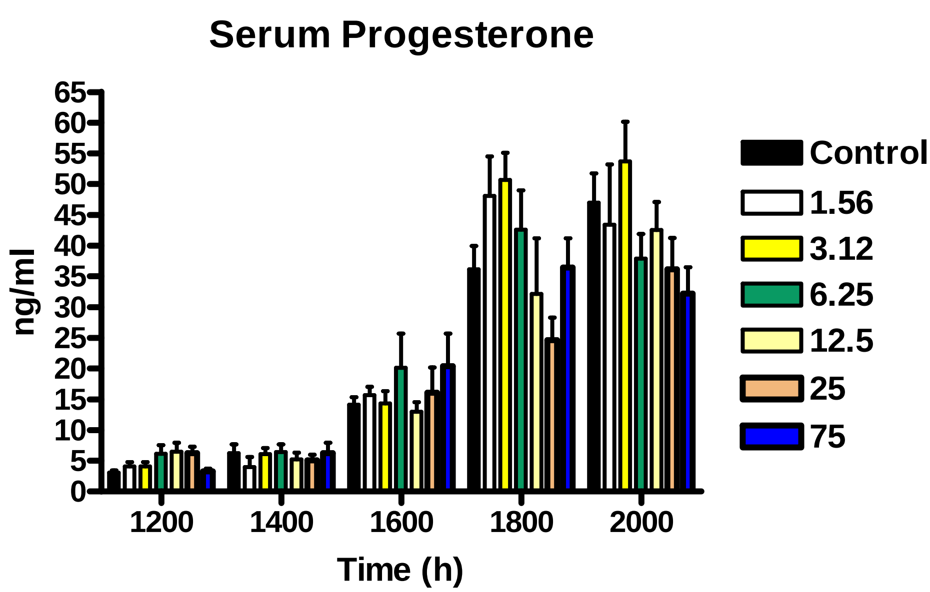

Figure 5. Serum progesterone at 1800 h.

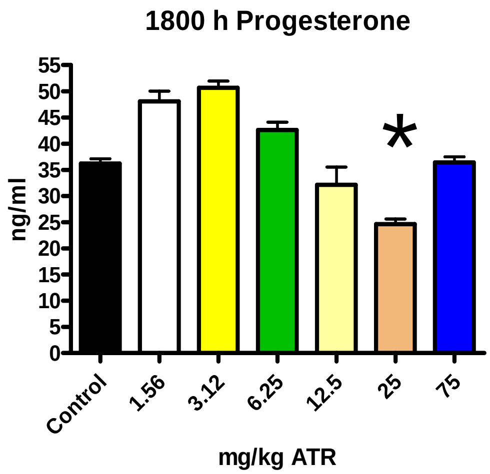

Figure 6

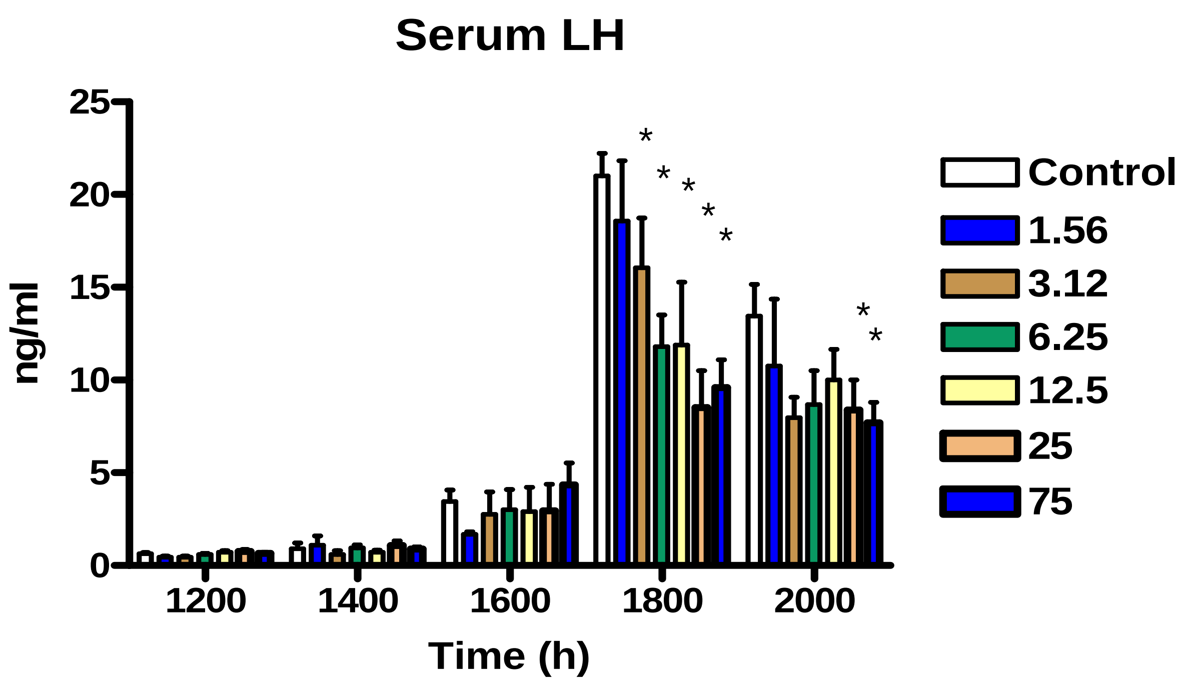

Figure 7.

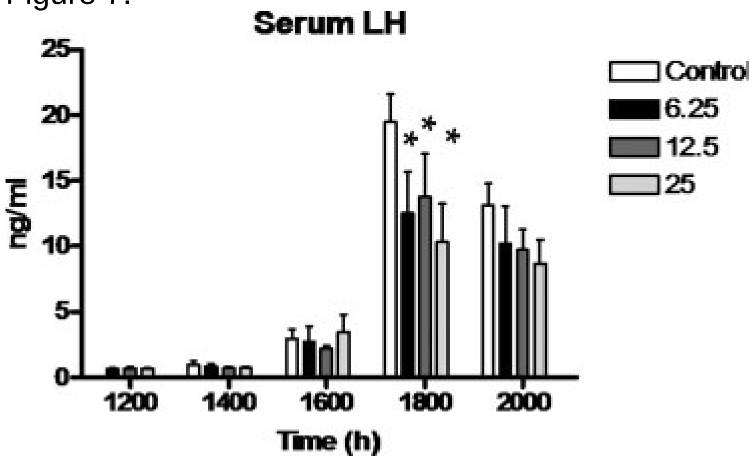

Figure 8.